

Macroalgae from S. Miguel Island as a potential source of antiproliferative and antioxidant products

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Ulva compressa, *Gelidium microdon*, *Osmundea pinnatifida*, *Fucus spiralis* and *Cystoseira abies-marina* from the coast of S. Miguel Island were screened for their *in vitro* cytotoxicity against HeLa tumour cell line, antioxidant potential and total phenolic content. From each alga, the hexane fraction (HF) of the methanol extract, the methanol residue (MF) and the dichloromethane extract (DE), were obtained and evaluated. The highest antiproliferative activity against HeLa cell line was found in *C. abies-marina* and *F. spiralis* DE (IC₅₀ 8.8 µg/mL and 10.7 µg/mL, respectively), and presented selective cytotoxicity, when tested against Vero cell line. Fluorescence microscopy studies of the two most active extracts suggest an apoptosis-inducing activity. Concerning the antioxidant activity, high values were found on *F. spiralis* MF and HF (EC₅₀ 0.62 µg/mL and 2.01 µg/mL, respectively), even higher than those obtained for trolox and quercetin. This high antioxidant activity in *F. spiralis* MF can be explained by its content in phenolic compounds, but not for HF, where the antioxidant compounds must be less polar. These results suggest that some macroalgae from the Azorean sea have great potential which could be considered for future applications in medicine, food production or cosmetics industry.

Key words: Azores marine macroalgae, cytotoxicity, HeLa, radical scavenging activity, total phenolic content

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INTRODUCTION

In recent years, macroalgae have increased their value to the pharmaceutical, *para*-pharmaceutical and food industries, due to their richness in new compounds with relevant biological activities and structural diversity (Hill 2011).

Although the Azores archipelago is rich in algal communities and some of them have been

used as food (e.g., *Fucus spiralis*, *Osmundea pinnatifida*) and in industry (e.g. *Gelidium microdon*) (Neto et al. 2006) only two papers have been published concerning the nutritional and pharmacological potential of this resource in the Azorean sea (Medeiros et al. 1999; Patarra et al. 2010). Namely, Medeiros et al. (1999) report that *Gelidium microdon*, *Fucus spiralis* and *Ulva rigida* presented *in vitro* cytotoxicity against A375

melanoma cell line. These species and others from the same genera are present in the intertidal habitat and are abundant throughout the year, which makes them ideal candidates for biotechnological applications and therefore for the present study. Another two species abundant in the Azorean coast were also selected as potentially interesting, *Cystoseira abies-marina* and *Osmundea pinnatifida*, for their resistance to predation (Granado & Caballero 2001; and personal observation by A.I. Neto) and also by reports, for *O. pinnatifida*, of antifungal and antibacterial activities (Rizvi & Shameel 2004).

In this work we report the screening of five species of macroalgae for their *in vitro* antitumour activity. Since radical scavenging activity is involved in fighting cancer and inflammation, antioxidant activity was also evaluated and the total phenolic content determined.

MATERIAL AND METHODS

ALGAE COLLECTION

The marine macroalgae were collected in the intertidal habitat in the south coast of S. Miguel, Azores, in the spring of 2007 and identified by A.I. Neto as *Ulva compressa* Linnaeus, *Gelidium microdon* Kützinger, *Osmundea pinnatifida* (Hudson) Stackhouse, *Fucus spiralis* Linnaeus and *Cystoseira abies-marina* (S.G. Gmelin) C. Agardh. Voucher samples of the algae are lodged in Ruy Telles Palhinha Herbarium (AZB), under the references SMG-07-18 (*C. abies-marina*), SMG-07-19 (*F. spiralis*), SMG-07-20 (*O. pinnatifida*), SMG-07-21 (*U.*) and SMG-07-22 (*G. microdon*).

PREPARATION OF THE EXTRACTS

Each alga was cleaned from salt and epiphytes using deionized water and stored at -20°C until extraction. A sample of each species (100 g fresh material) was ground with liquid nitrogen and was sequentially extracted (1:4 biomass:solvent ratio, g/mL) at room temperature, for 8h, with methanol and dichloromethane (DE extract). The methanol extract was partitioned with *n*-hexane, yielding the hexane (HF) and methanol (MF) fractions. The extract (DE) and fractions (MF and HF) were evaporated to dryness in vacuum. For the bioassays and other determinations, aliquots

of the dry samples were dissolved in DMSO to a final concentration of 50 mg/mL.

CYTOTOXICITY ASSAYS

In vitro cytotoxicity against HeLa tumour cell line (human cervix carcinoma, ATCC) was assessed using the colorimetric MTT reduction assay with the cells in lag phase, as described previously (Moujir et al. 2008). Non-tumour Vero cell line (African Green Monkey kidney, ATCC), was used to assess cytotoxic selectivity. Results of at least three experiments were expressed as IC₅₀ values.

For fluorescence microscopy, HeLa cells were grown for 24h in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2% Fetal Bovine Serum (FBS). Each well of a 24-well ELISA plate, containing a polylysine-treated cover slip, was seeded with 400 µL of 4x10⁵ cells/mL DMEM. After 24h, DMEM was replaced by fresh medium or with medium containing extract or fractions in the appropriate dilutions. After 12h, BCECF and Hoechst 33342 (Sigma) dyes were added. Preparations were visualized in a Nikon Eclipse 80 i fluorescence microscope, with a 488 nm (BCECF) or 461 nm filter (Hoechst).

DPPH RADICAL SCAVENGING ASSAY

Antioxidant activity was assayed by the DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging assay (Blois 1958). Algae extracts, fractions or reference compounds (trolox, BHT (butylated hydroxytoluene), quercetin and ascorbic acid) were added to a methanolic DPPH solution at different concentrations and the absorbance at 517 nm was measured after 30 min in the dark. In each assay, a control was prepared, in which the sample or standard was substituted by the same amount of solvent. Percentage of antioxidant activity (%AA) was calculated as

$$\% \text{ AA} = 100 [1 - (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}]$$

where, A_{control} is the absorbance of the control and A_{sample} is the absorbance of the extract or standard. All assays were carried out in triplicate and results expressed as EC₅₀, *i.e.*, as the concentration yielding 50% scavenging of DPPH, calculated by interpolation from the % AA vs concentration curve.

TOTAL PHENOLIC CONTENT (PC)

The content of total phenolic compounds in crude extracts and each fraction was determined by a colorimetric reaction using Folin & Ciocalteu's phenol reagent (Kuda et al. 2005). TPC values were expressed as μg gallic acid equivalents/mg extract.

RESULTS AND DISCUSSION

The extraction yields showed great variability (Table 1), depending on solvent polarity and macroalgae species. A great variability in yield of extraction is also reported in the literature (Jiménez-Escrig et al. 2011). The higher extraction yields were obtained with methanol. Two species, *O. pinnafitida* and *U. compressa*, stand out as having the lowest extraction yields for all the solvents. The cytotoxicity of the DE, HF and MF from the macroalgae *C. abies-marina*, *F. spiralis*, *G. microdon*, *O. pinnafitida* and *U.*

compressa against HeLa and Vero cell lines are shown on Table 2. Only two of the fifteen tested samples displayed no cytotoxicity against HeLa cell line ($\text{IC}_{50} > 200 \mu\text{g/mL}$). The strongest activities were found in the dichloromethane extracts, particularly on extracts of *C. abies-marina*, *F. spiralis* and *U. compressa* with IC_{50} values of 8.8, 10.7 and 17.8 $\mu\text{g/mL}$, respectively, therefore meeting the NCI (National Cancer Institute) criteria for considering a crude extract as active (Boyd 1997).

Table 1. Extraction yield of the samples evaluated (g of dried extract/ 100 g of the starting fresh algae); MF, methanol fraction; DE, dichloromethane extract; and HF, Hexane fraction.

| Algae | MF | DE | HF |
|--------------------------------|-------|--------|-------|
| <i>Cystoseira abies-marina</i> | 1.82 | 0.185 | 0.188 |
| <i>Fucus spiralis</i> | 1.14 | 0.205 | 0.191 |
| <i>Gelidium microdon</i> | 2.41 | 0.0980 | 0.193 |
| <i>Osmundea pinnatifida</i> | 0.446 | 0.0556 | 0.031 |
| <i>Ulva compressa</i> | 0.750 | 0.160 | 0.125 |

Table 2. Cytotoxicity, DPPH radical scavenging activity and total phenolic content of the macroalgae extracts and fractions. ^a MF - methanol fraction, DE - dichloromethane extract, HF - Hexane fraction; ^b Cytotoxicity, Taxol was used as a positive control ($\text{IC}_{50} = 0.088 \pm 0.012$ against HeLa and 1.46 ± 0.16 against Vero); ^c DPPH radical scavenging activity: Trolox ($\text{EC}_{50} = 5.98 \pm 0.18$), BHT ($\text{EC}_{50} = 31.04 \pm 0.96$), quercetin ($\text{EC}_{50} = 3.07 \pm 0.01$) and ascorbic acid ($\text{EC}_{50} = 10.32 \pm 0.01$) were used as references; ^d Total phenolic content is expressed as μg GAE (Gallic Acid Equivalents) / mg extract or fraction.

| Macroalgae | a | Cytotoxicity, IC_{50} ($\mu\text{g/mL}$) ^b | | DPPH scavenging activity, EC_{50} ($\mu\text{g/mL}$) ^c | Total phenolic content ^d |
|--------------------------------|----|--|-------------------|--|-------------------------------------|
| | | HeLa | Vero | | |
| <i>Cystoseira abies-marina</i> | MF | >200 | >200 | >500 | 135.08 \pm 1.572 |
| | DE | 8.8 \pm 1.29 | 48.2 \pm 1.60 | 329.5 \pm 18.61 | 14.21 \pm 1.237 |
| | HF | 26.3 \pm 2.06 | 47.8 \pm 2.75 | 137.2 \pm 8.26 | 6.23 \pm 2.52 |
| <i>Fucus spiralis</i> | MF | 127.2 \pm 3.65 | >200 | 0.62 \pm 0.01 | 184.04 \pm 11.614 |
| | DE | 10.7 \pm 1.71 | 84.8 \pm 3.19 | >500 | 13.02 \pm 1.49 |
| | HF | 55.5 \pm 7.72 | 131.0 \pm 18.26 | 2.06 \pm 0.18 | 3.10 \pm 0.722 |
| <i>Gelidium microdon</i> | MF | 130.3 \pm 4.57 | >200 | >500 | 28.00 \pm 1.250 |
| | DE | 88.4 \pm 7.69 | 161.9 \pm 10.56 | >500 | 19.21 \pm 0.714 |
| | HF | 98.4 \pm 5.53 | >200 | >500 | 6.23 \pm 2.526 |
| <i>Osmundea pinnatifida</i> | MF | 173.7 \pm 14.84 | >200 | >500 | 34.67 \pm 0.361 |
| | DE | 129.3 \pm 17.79 | >200 | >500 | 33.26 \pm 2.296 |
| | HF | >200 | >200 | >500 | 2.27 \pm 0.722 |
| <i>Ulva compressa</i> | MF | 45.5 \pm 9.38 | >200 | >500 | 29.86 \pm 1.250 |
| | DE | 17.8 \pm 4.17 | 28.1 \pm 1.21 | >500 | 15.17 \pm 3.934 |
| | HF | 169.8 \pm 11.42 | 118.9 \pm 1.75 | >500 | 24.77 \pm 0.722 |

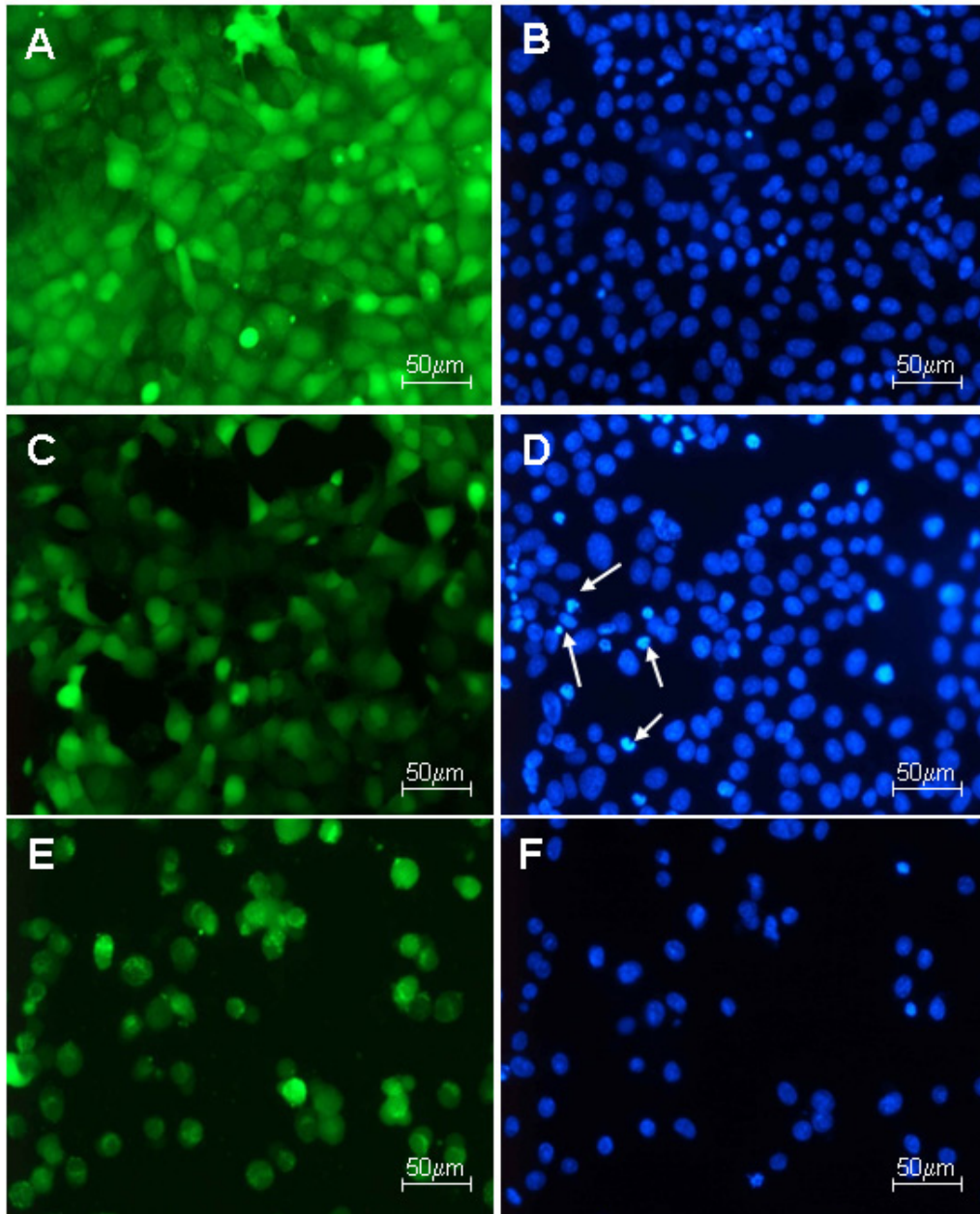


Fig. 1. Fluorescence microscopy of HeLa cells (on the left, BCECF cytoplasmic dye and on the right, Hoechst 33342 nuclear dye). A and B, control, C and D, cells exposed to 17 $\mu\text{g/mL}$ DE-*Cystoseira* extract, E and F, cells exposed to 23.6 $\mu\text{g/mL}$ DE-*Fucus* extract), respectively; arrows indicate chromatin condensation.

Recently, Mhadhebi et al. (2011, 2012) also showed the great potential of extracts from other *Cystoseira* species as antiproliferative agents against different tumour cell lines. The methanol fractions showed the lowest antiproliferative effect (except in the case of *U. compressa*, where the MF was more active than the HF fraction). It is important to emphasize that the tested extracts and fractions have selective cytotoxicity, since HeLa cells were more affected than Vero (for the most active extracts, the IC₅₀ values for Vero were 6-8 times higher than for HeLa cell line).

The two most active extracts (DE from *F. spiralis* and *C. abies-marina*) were selected for fluorescence microscopy with BCECF and Hoechst dyes, in order to detect morphological alterations in the target cells which might contribute to elucidate the mechanisms involved in cytotoxicity by these extracts. Fluorescence images show the dramatic alterations in cell shape and in the nuclei of cells treated for 12h with DE from *Fucus* or *Cystoseira* at 2xIC₅₀ concentration (Fig. 1). The presence of chromatin condensation and/or fragmentation (see white arrows on Fig 1) cell rounding and shrinking, and membrane-contained cytoplasmic vesicles indicate apoptosis (Elmore 2007).

The antioxidant activity of all the samples was evaluated by the DPPH assay (Table 2). The MF and HF from *F. spiralis* extracts stand out as extremely active (EC₅₀ 0.62 ± 0.01 and 2.06 ± 0.18 µg/mL). They have a stronger free radical-scavenging capacity than trolox, quercetin, ascorbic acid and BHT, used as positive controls. The highest radical-scavenging activity of MF from *F. spiralis* is coincident with the highest total phenolic content and could be explained, at least partially, by the presence of phlorotannins of the fucol and fucophlorethol classes (Cerantola et al. 2006). The high radical-scavenging activity of HF cannot be due to phenolic compounds but to less polar compounds, such as carotenoids or tocopherol derivatives, as referred by Burtin (2003).

The present findings showed: a) the selective and antiproliferative activity, induced by apoptosis, of the dichloromethane extract from *C. abies-marina*; b) the higher antioxidant activity of methanol and hexane fractions from *F. spiralis*; c) the potential of *C. abies-marina* and *F. spiralis*

from the Azorean sea for the pharmaceutical, nutraceutical, food and/or cosmetics industry. These species will be further investigated in order to recognize and characterize the specific compounds responsible for the activities observed.

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